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Effect of Incorporation of Cidofovir into DNA by Human Cytomegalovirus DNA Polymerase on DNA Elongation

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Cidofovir (CDV) (HPMPC) has potent in vitro and in vivo activity against human cytomegalovirus (HCMV). CDV diphosphate (CDVpp), the putative antiviral metabolite of CDV, is an inhibitor and an alternate substrate of HCMV DNA polymerase. CDV is incorporated with the correct complementation to dGMP in the template, and the incorporated CDV at the primer end is not excised by the 3′-to-5′ exonuclease activity of HCMV DNA polymerase. The incorporation of a CDV molecule causes a decrease in the rate of DNA elongation for the addition of the second natural nucleotide from the singly incorporated CDV molecule. The reduction in the rate of DNA (36-mer) synthesis from an 18-mer by one incorporated CDV is 31% that of the control. However, the fidelity of HCMV DNA polymerase is maintained for the addition of the nucleotides following a single incorporated CDV molecule. The rate of DNA synthesis by HCMV DNA polymerase is drastically decreased after the incorporation of two consecutive CDV molecules; the incorporation of a third consecutive CDV molecule is not detectable. Incorporation of two CDV molecules separated by either one or two deoxynucleoside monophosphates (dAMP, dGMP, or dTMP) also drastically decreases the rate of DNA chain elongation by HCMV DNA polymerase. The rate of DNA synthesis decreases by 90% when a template which contains one internally incorporated CDV molecule is used. The inhibition by CDVpp of DNA synthesis by HCMV DNA polymerase and the inability of HCMV DNA polymerase to excise incorporated CDV from DNA may account for the potent and long-lasting anti-CMV activity of CDV.

Human cytomegalovirus (HCMV) is a member of the herpesviruses and can cause life- and sight-threatening infections in immunocompromised patients. One of the HCMV-encoded proteins is DNA polymerase, comprised of two polypeptides with molecular masses of 140,000 and 58,000 Da (12). HCMV DNA polymerase is the target of three antiviral agents in clinical use: ganciclovir [9-(1,3-dihydroxy-2-propoxymethyl) guanine [GCV]), phosphonoformate, and cidofovir (CDV) (8, 14, 15). GCV is a potent inhibitor of HCMV replication (10). Selective phosphorylation of GCV is initiated by the HCMV-encoded UL97 kinase (1). Additional phosphorylations of GCV monophosphate by cellular kinases to GCV triphosphate are required for inhibition of HCMV DNA polymerase-directed DNA synthesis (14). GCV is a nonobligatory DNA chain terminator after its incorporation into DNA catalyzed by both herpes simplex virus type 1 DNA polymerase and DNA polymerase α (9, 14). The incorporation of GCV monophosphate into DNA by herpes simplex virus type 1 DNA polymerase results in a significant reduction in V_{max} for subsequent nucleotide incorporations (13). Phosphonoformate is a pyrophosphate analog and inhibits HCMV DNA polymerase without prior activation by cellular enzymes (8).

CDV is an acyclic cytidine phosphonate analog which is mechanistically different from GCV. CDV is resistant to phosphatase and is not dependent on phosphorylation by UL97 for its activity against HCMV (7). Thus, clinical isolates resistant to GCV, the majority of which have mutations in UL97, remain susceptible to CDV (11). In this study, we investigated the consequence of incorporation of CDV on the rate and fidelity of DNA elongation catalyzed by HCMV DNA polymerase.

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MATERIALS AND METHODS

Chemicals. Heparin-agarose and single-stranded-DNA–agarose were from GIBCO-BRL. Activated calf thymus DNA was from Pharmacia. CDV diphosphate (CDVpp) was synthesized as previously described (5). [^{3}H]dTTP (81 Ci/mmol) and [^{3}H]dCTP (25 Ci/mmol) were from New England Nuclear. [γ-^{32}P]ATP (5,000 Ci/mmol) was from Amersham. DE52 cellulose and P-11 cellulose were from Whatman. All other chemicals were of the highest grades commercially available.

Purification of HCMV DNA polymerase. Normal human dermal fibroblasts were infected with HCMV (Towne strain) at a multiplicity of infection of 0.005 and harvested when 60% of the cells showed cytopathic effect (approximately 4 days). HCMV DNA polymerase was purified through four successive chromatographic steps (DEAE-cellulose, phosphocellulose, heparin-agarose, and single-stranded-DNA–agarose) as described previously (12), with the following modification: only those fractions containing DNA polymerase activities which could be stimulated by 90 mM $(NH_4)_2SO_4$ were pooled for further purification (12). For some preparations, in order to stabilize the enzyme, column eluates were collected into tubes containing bovine serum albumin (BSA) at a final concentration of 0.2 mg/ml. All buffer solutions contained 10% glycerol. The purified HCMV DNA polymerase could be activated 448% by 90 mM $(NH_4)_2SO_4$. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified HCMV DNA polymerase showed two protein bands corresponding to molecular masses of 140,000 and 58,000 Da (12). The identity of the purified HCMV DNA polymerase was further confirmed by Western blot analysis with antibody to the 140-kDa subunit. The addition of BSA to the purified HCMV DNA polymerase was found to greatly increase the stability of the enzyme. The activity of the purified HCMV DNA polymerase during the assay stayed linear for at least 90 min. The purified HCMV DNA polymerase was stored at −70°C until use.

Enzyme assays. DNA polymerases from HCMV and human cells were assayed as described previously (3, 4, 6). During enzyme purification, the DNA polymerase assays were monitored by the measurement of the incorporation of a tritium-labeled nucleoside triphosphate into an acid-precipitable product. Briefly, the 50-μl assay solution contained 5% glycerol; 10 mM $MgCl_2$; 0.4 mM dithiothreitol (DTT); 20 mM Tris (pH 8.0); 100 μM (each) dATP, dGTP, and dCTP; 5μM [^{3}H]TTP (specific activity, 4 Ci/mmol); 10 μg of activated calf thymus DNA; and, where indicated, 90 mM $(NH_4)_2SO_4$ (12). Aliquots were taken at various times during the incubation and spotted onto Whatman 3MM filter paper discs (2 cm in diameter). The paper discs were washed three times in 5% trichloroacetic acid–1% pyrophosphate and once in 95% ethanol. After drying, the radioactivities on the discs were measured in a Beckman counter with Beckman Ready Safe scintillation fluid. One unit of HCMV DNA polymerase was defined as the amount of enzyme catalyzing the incorporation of 1 pmol of dTMP/min at 37°C. The kinetic analysis of CDVpp interaction with HCMV DNA polymerase was carried out as described previously (2).

A

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Primer:    SX1   5' TGACCATGTAACAGAGAG 3'

Template:  SX2   3' ACTGGTACATTGTCTCTCGTTCTCTCTCTCTTCTCT 5'
           SX3   3' ACTGGTACATTGTCTCTCGGTCTCTCTCTCTTCTCT 5'
           SX7   3' ACTGGTACATTGTCTCTCATTCTCTCTCTCTTCTCT 5'
           SX8   3' ACTGGTACATTGTCTCTCCTTCTCTCTCTCTTCTCT 5'
           SX9   3' ACTGGTACATTGTCTCTCTTTCTCTCTCTCTTCTCT 5'
           SX29  3' ACTGGTACATTGTCTCTCGTGCTCTCTCTCTTCTCT 5'
           SX30  3' ACTGGTACATTGTCTCTCGAGCTCTCTCTCTTCTCT 5'
           SX31  3' ACTGGTACATTGTCTCTCGCGCTCTCTCTCTTCTCT 5'
           SX35  3' ACTGGTACATTGTCTCTCGATGTCTCTCTCTTCTCT 5'
           SX36  3' ACTGGTACATTGTCTCTCGCAGTCTCTCTCTTCTCT 5'
           SX37  3' ACTGGTACATTGTCTCTCGCTGTCTCTCTCTTCTCT 5'
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B

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SX18 5' GAGGTCGACGAATTC 3'
                    NlaIII
          SX1 5'TGACCATGTAACAGAGAG 3'

SX14 3' CTCCAGCTGCTTAAGACTGGTACATTGTCTCTCGTTCTCTCTCTCTTCTCTCACTTCCACCATCAA 5'

                                        SX27 3' TTCTCTCACTTC 5'
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FIG. 1. (A) Primers and templates used in this study. Boldface indicates the nucleotide sequences designed for base pairing with CDV. (B) *Nla*III site.

Synthesis of the CDV terminated primer. ^{32}P-, 5′-end-labeled synthetic primer SX1 (5′-TGA-CCA-TGT-AAC-AGA-GAG-3′; 150 pmol) was annealed to synthetic template SX2 (5′-TCT-CTT-CTC-TCT-CTC-TTG-CTC-TCT-GTT-ACA-TGG-TCA-3′). Incorporation of one molecule of CDV at the 3′ end of SX1 was carried out in a 60-μl reaction mixture containing 20 mM Tris (pH 8.0), 10 mM $MgCl_2$, 0.2 mg of BSA per ml, 0.4 mM DTT, 90 mM $(NH_4)_2SO_4$, 50 μM CDVpp, and 1.2 U of HCMV DNA polymerase. After overnight incubation at 37°C, the reaction mixture was loaded onto a 15% polyacrylamide–8 M urea gel. After electrophoresis the gel was visualized by autoradiography. The gel slice containing a 19-mer with one CDV molecule at the 3′ terminus was excised and put into 0.2 ml of gel elution buffer (0.1% sodium dodecyl sulfate, 0.5 M ammonium acetate, and 10 mM $MgCl_2$). Elution was carried out at 37°C for 4 h. The gel eluate was transferred to a clean tube and extracted with *n*-butanol to reduce the volume to less than 50 μl. The concentrated gel eluate was loaded on a G-25 quick-spin column (Boehringer Mannheim) to remove salts from the sample. The CDV terminated primer was quantitated by measuring the ^{32}P radioactivity in a scintillation counter.

The CDV terminated primer was used to study the 3′-to-5′ exonuclease activity of HCMV DNA polymerase.

Synthesis and purification of a DNA template containing one single internally incorporated CDV. Two hundred picomoles of an 18-mer primer, SX1, annealed to a 66-mer template, SX14 (5′ AAC-TAC-CAC-CTT-CAC-TCT-CTT-CTC-

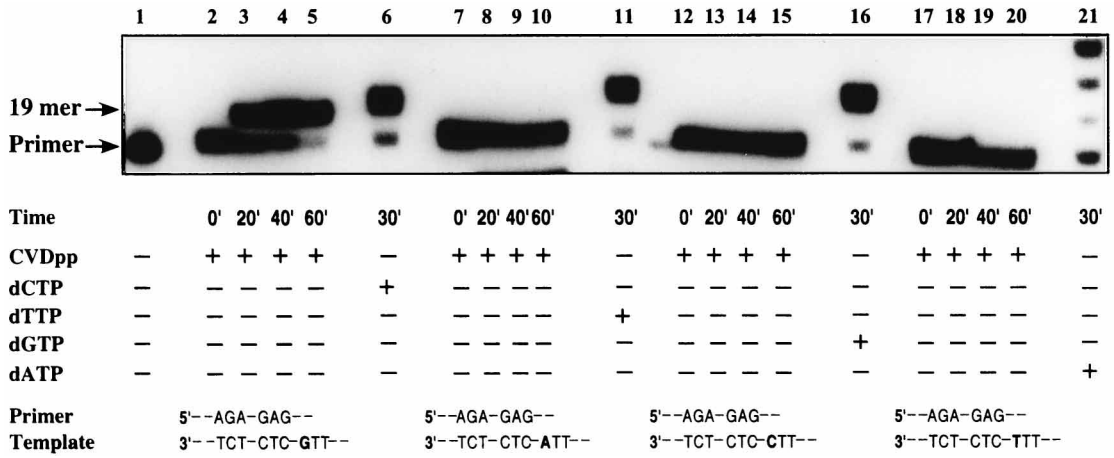

FIG. 2. Primer extension analysis of the fidelity of HCMV DNA polymerase during incorporation of CDVpp into DNA. An 18-mer ^{32}P-, 5′-end-labeled oligonucleotide, SX1 (5′ TGA-CCA-TGT-AAC-AGA-GAG 3′), was annealed to one of the following 36-mer templates: SX2 (5′ TCT-CTT-CTC-TCT-CTC-T**T**G-CTC-TCT-GTT-ACA-TGG-TCA 3′ [lanes 1 to 6]) SX7 (5′ TCT-CTT-CTC-TCT-CTC-TT**A**-CTC-TCT-GTT-ACA-TGG-TCA 3′ [lanes 7 to 11]), SX8 (5′ TCT-CTT-CTC-TCT-CTC-TT**C**-CTC-TCT-GTT-ACA-TGG-TCA 3′ [lanes 12 to 16]), or SX9 (5′ TCT-CTT-CTC-TCT-CTC-TT**T**-CTC-TCT-GTT-ACA-TGG-TCA 3′ [lanes 17 to 21]). The experimental details are described in Materials and Methods. CDVpp was used at 100 μM. dATP, dCTP, dGTP, and dTTP, when present, were at 50 μM. Samples were taken from each of the reaction mixtures at the indicated times (in minutes). Control reaction mixtures contained 50 μM dCTP, dTTP, dGTP, or dATP. A single sample was taken from each of these reaction mixtures. Boldface indicates the nucleotide sequences designed for base pairing with CDV.

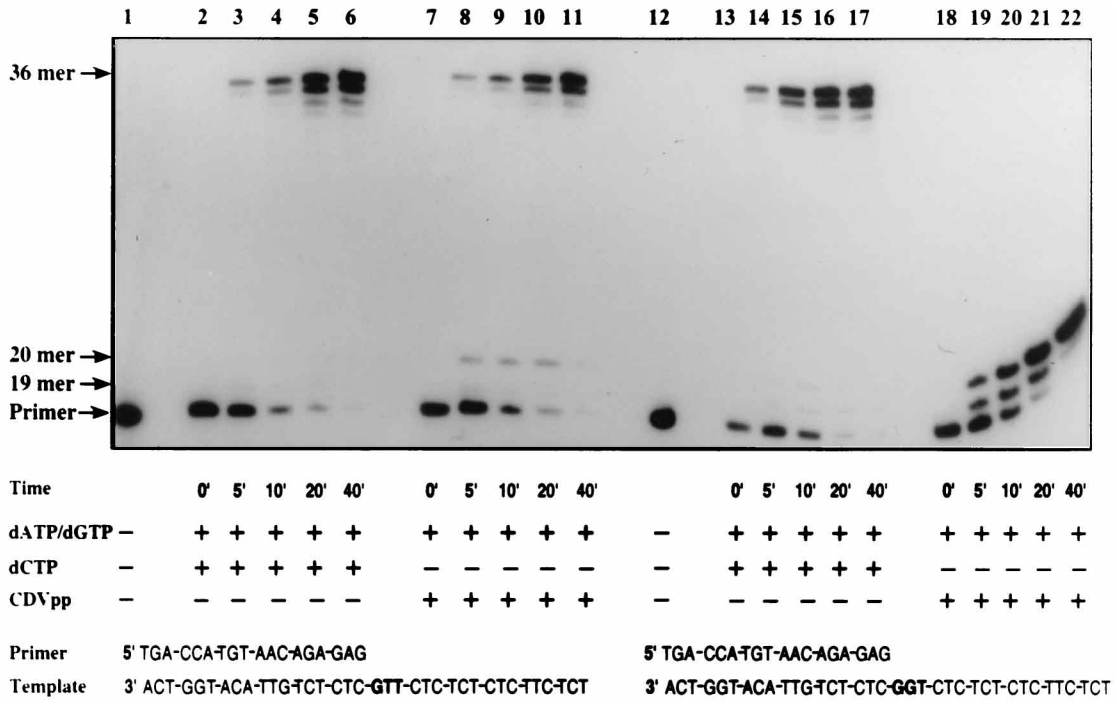

FIG. 3. Effects of CDVpp on DNA strand elongation catalyzed by HCMV DNA polymerase with templates containing one G or two consecutive Gs in the coding sequences. An 18-mer ^{32}P-, 5′-end-labeled oligonucleotide, SX1, was annealed to one of the 36-mer templates SX2 (lanes 1 to 11) and SX3 (lanes 12 to 22). The experimental details are described in Materials and Methods. All reaction mixtures contained 50 μM dATP or dGTP. Control reaction mixtures also contained 50 μM dCTP. CDVpp was used at 100 μM. Samples were taken from each reaction mixture at the indicated times (in minutes) after the addition of enzyme. Boldface indicates the nucleotide sequences designed for base pairing with CDV.

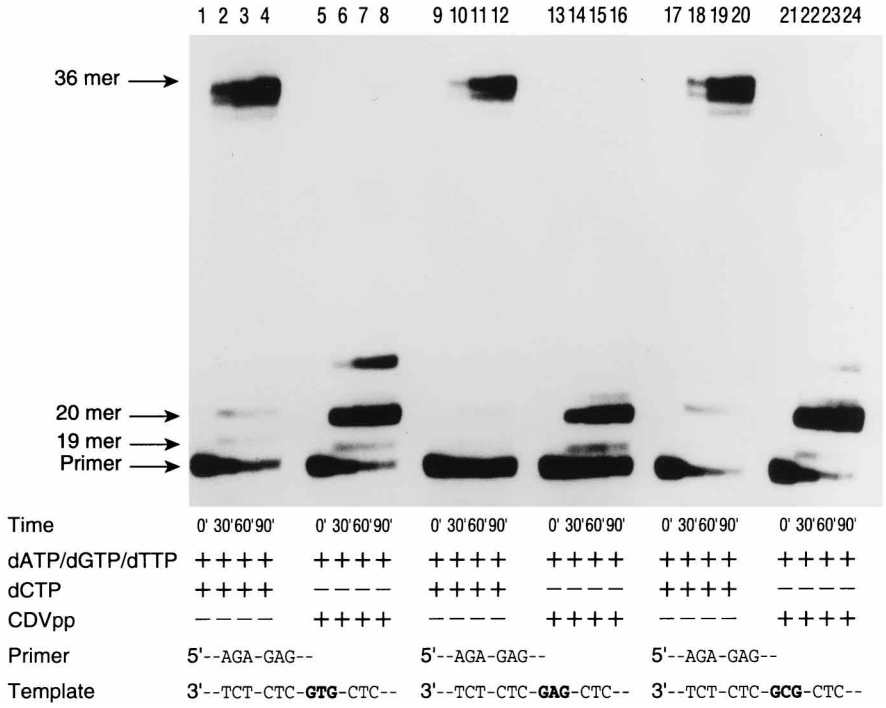

FIG. 4. Effects of CDVpp on DNA strand elongation catalyzed by HCMV DNA polymerase with templates containing two Gs separated by one T, one A, or one C in the coding sequences (indicated by boldface). An 18-mer ^{32}P-, 5′-end-labeled oligonucleotide, SX1, was annealed to one of the following 36-mer templates: SX29 (5′ TCT-CTT-CTC-TCT-CTC-**GTG**-CTC-TCT-GTT-ACA-TGG-TCA 3′ [lanes 1 to 8]), SX30 (5′ TCT-CTT-CTC-TCT-CTC-**GAG**-CTC-TCT-GTT-ACA-TGG-TCA 3′ [lanes 9 to 16]), or SX31 (5′ TCT-CTT-CTC-TCT-CTC-**GCG**-CTC-TCT-GTT-ACA-TGG-TCA 3′ [lanes 17 to 24]). The experimental details are described in Materials and Methods. dATP, dGTP, and dTTP were used at 50 μM. Control reaction mixtures also contained 50 μM dCTP. CDVpp was used at 100 μM. Samples were taken from each reaction mixture at the indicated times (in minutes) after the addition of enzyme.

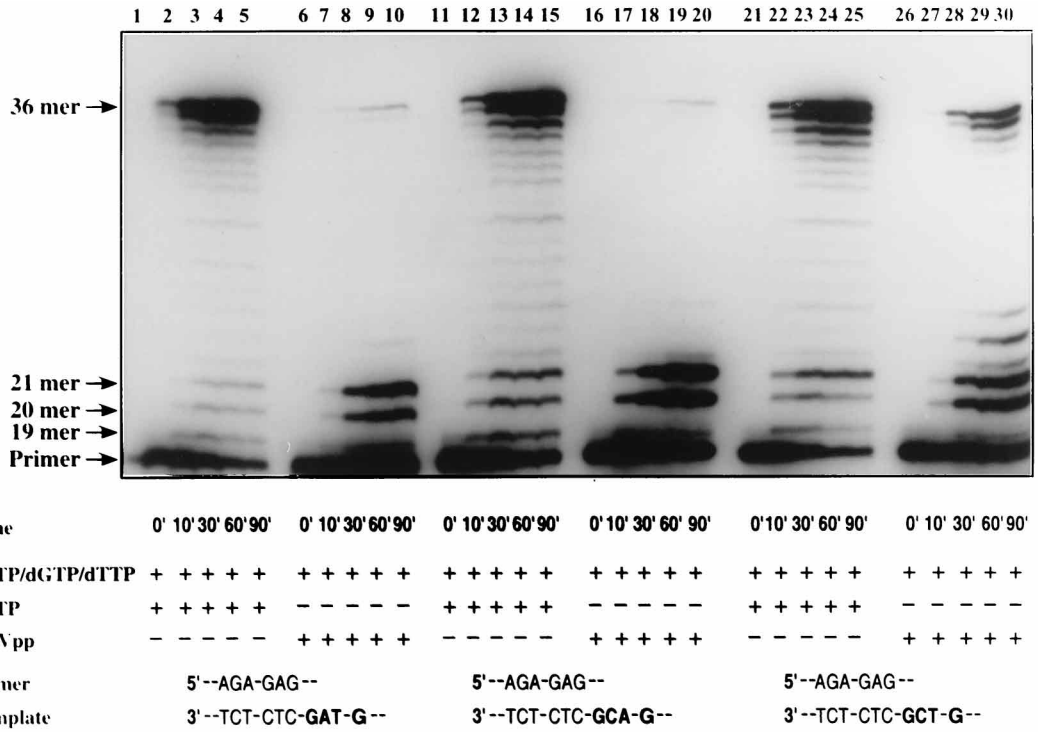

FIG. 5. Effects of CDVpp on DNA strand elongation catalyzed by HCMV DNA polymerase with templates containing two Gs separated by two nucleotides (TA, AC, or TC) in the coding sequences (indicated by boldface). An 18-mer ^{32}P-, 5′-end-labeled oligonucleotide, SX1, was annealed to one of the following 36-mer templates: SX35 (5′ TCT-CTT-CTC-TCT-CT**G**-TA**G**-CTC-TCT-GTT-ACA-TGG-TCA 3′ [lanes 1 to 10]), SX36 (5′ TCT-CTT-CTC-TCT-CT**G**-AC**G**-CTC-TCT-GTT-ACA-TGG-TCA 3′ [lanes 11 to 20]), or SX37 (5′ TCT-CTT-CTC-TCT-CT**G**-TC**G**-CTC-TCT-GTT-ACA-TGG-TCA 3′ [lanes 21 to 30]). The experimental details are described in Materials and Methods. dATP, dGTP, and dTTP were used at 50 μM. Control reaction mixtures also contained 50 μM dCTP. CDVpp was used at 100 μM. Samples were taken from each reaction mixture at the indicated times (in minutes) after the addition of enzyme.

TCT-CTC-TTG-CTC-TCT-GTT-ACA-TGG-TCA-GAA-TTC-GTC-GAC-CTC3′), was used as the primer-template for making a 51-base single-stranded DNA template containing one internally incorporated CDV molecule. The reaction was carried out in 200 μl of reaction buffer containing 20 mM Tris (pH 8.0); 10 mM $MgCl_2$; 0.2 mg of BSA per ml; 0.4 mM DTT; 90 mM $(NH_4)_2SO_4$; 100 μM (each) dATP, dGTP, and dTTP; 50 μM CDVpp; and 7.2 U of HCMV DNA polymerase. A 15-mer oligonucleotide, SX18 (5′ GAG-GTC-GAC-GAA -TCC 3′), complementary to the 3′ end region of SX14 was also included in the reaction mixture to prevent the degradation of the 3′ single-stranded region of SX14 by the intrinsic 3′-to-5′ exonuclease activity of HCMV DNA polymerase. After overnight incubation at 37°C, the reaction mixture was loaded onto a 15% polyacrylamide–8 M urea gel. The 51-base single-stranded DNA containing one internally incorporated CDV molecule was purified as described in "Synthesis of the CDV terminated primer."

A 43-mer template containing CDV was synthesized by primer elongation of SX1-SX14 with HCMV DNA polymerase as described above. The DNA strand containing one internally incorporated CDV molecule was separated from the other strand by restriction digestion with *Nla*III. The resulting 43-mer CDV-containing DNA strand was gel purified as described above.

The primers and templates used in this study are listed in Fig. 1.

In vitro DNA synthesis with 5′-end-labeled primers. Synthetic primers were phosphorylated with [γ-^{32}P]ATP by using T4 polynucleotide kinase (New England Biolabs). The end-labeled primers were purified by passage through a quick-spin column (Sephadex G-25; Boehringer Mannheim Biochemicals, Inc.). ^{32}P-labeled primers were annealed to templates at a 1:2 molar ratio of primer to template in HCMV DNA polymerase buffer [20 mM Tris (pH 8.0), 10 mM $MgCl_2$, 0.2 mg of BSA per ml, 0.4 mM DTT, and 90 mM $(NH_4)_2SO_4$] by incubating the mixture at 65°C for 5 min and allowing it to cool to room temperature slowly. The HCMV DNA polymerase reaction mixture (20 μl) contained 20 mM Tris (pH 8.0), 10 mM $MgCl_2$, 0.2 mg of BSA per ml, 0.4 mM DTT, 90 mM $(NH_4)_2SO_4$, 0.05 μM ^{32}P-labeled primer, 0.1 μM template, and 0.096 U of HCMV DNA polymerase. dATP, dGTP, and dTTP, when present, were at 50 μM, whereas CDVpp or dCTP was present at various concentrations. Reaction mixtures were incubated at 37°C. Samples were removed at various times and put into equal volumes of stop solution (95% formamide). The products of DNA synthesis were separated by gel electrophoresis on a 15% polyacrylamide–8 M urea gel. After electrophoresis, the gel was visualized by autoradiography and examined with an Ambis image acquisition and analysis scanner.

RESULTS

Fidelity in the incorporation of CDV. The fidelity of CDV incorporation is shown in Fig. 2. CDV was incorporated only opposite dG; no incorporation was observed opposite dA, dC, or dT.

Effect of CDVpp on DNA elongation. As shown in Fig. 3, in the presence of natural nucleotide substrates HCMV DNA polymerase catalyzes the DNA elongation from an 18-mer to a 36-mer rapidly with either one dG (lanes 2 to 6) or two consecutive dGs (lanes 13 to 17) in the template. With one dG in the template the substitution of CDVpp for dCTP results in the transient appearance of a 20-mer, suggesting that the polymerase pauses following incorporation of CDV (lanes 7 to 11). The overall rate reduction in the synthesis of a full-length 36-mer caused by incorporation of one molecule of CDV is 31%. Also, DNA elongation is dramatically reduced or terminated after the incorporation of two consecutive molecules of CDV (lanes 18 to 22).

The effect on the kinetics of DNA elongation following incorporation of two CDV molecules separated by one molecule of natural nucleotide (dAMP, dGMP, or dTMP) was examined. As shown in Fig. 4, the elongation of DNA to a full-length 36-mer was observed for the control samples in the presence of dCTP for all three template sequences (lanes 1 to 4, 9 to 12, and 17 to 20). In contrast, no full-length products were observed in the presence of CDVpp in all three instances

(Fig. 4, lanes 5 to 8, 13 to 16, and 21 to 24), with some differences in the accumulation of DNA with various sizes. The accumulation of mainly a 20-mer was observed with the template sequences of GAG (Fig. 3, lanes 13 to 16) and GCG (Fig. 4, lanes 21 to 24). The accumulation of predominately a 20-mer and 22-mer were observed for the template sequence of GTG (lanes 5 to 8).

Figure 5 shows the effect of incorporation of two CDV molecules separated by two molecules of natural nucleotide (dAMP, dGMP, or dTMP). The reductions in the rates of formation of DNAs (25- to 36-mers) were 96, 95, and 74% for the templates with two Gs separated by AT, CA, and CT, respectively.

Fidelity of HCMV DNA polymerase following the incorporation of CDV and effect of a CDV-containing template on DNA elongation. We next examined the fidelity of DNA synthesis following the incorporation of a single molecule of CDV at the elongating primer end. As shown in Fig. 6A, the sequence of the CDV-containing DNA was as expected based on the sequence of the template used for its synthesis by HCMV DNA polymerase. Figure 6A also shows that during the sequencing (T7 polymerase) reaction the accumulation of DNA at ddATP and ddGTP could be observed at the position complementary to CDV in the template. As shown in Fig. 6b (lane 2), the rate of DNA synthesis was slowed down by 90%, with an apparent pausing at the CDV location on the template.

HCMV DNA polymerase does not excise CDV from the primer containing one molecule of CDV at the 3′ terminus. In the absence of other deoxynucleoside triphosphates the 3′-to-5′ exonuclease activity of the HCMV DNA polymerase can be measured easily. Figure 7 shows the 3′-to-5′ exonuclease activity of the HCMV DNA polymerase on primers containing one molecule of either dCMP or CDV. As shown in Fig. 7 (lanes 8 to 14), dCMP can be excised from the mismatched 3′ primer end whereas no removal of CDV when base paired to dG was observed (Fig. 7, lanes 1 to 7).

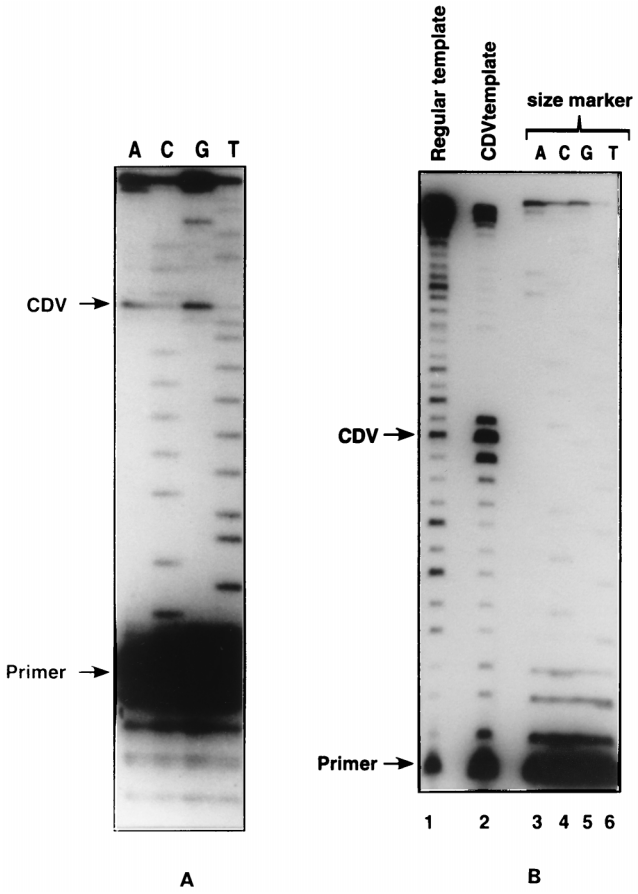

FIG. 6. Determination of the fidelity of a template containing one internally incorporated CDV and primer extension analysis of the capability of HCMV DNA polymerase to read through a DNA template containing one internally incorporated CDV molecule. (A) A 24-mer ^{32}P-, 5-end-labeled oligonucleotide, SX15 (5′-GCC-AGT-ACG-AAC-TAC-CAC-CTT-CAC-3′), was used to sequence a 43-mer template containing one internally incorporated CDV molecule. The 43-mer template was synthesized by HCMV DNA polymerase as described in Materials and Methods. (B) A 12-mer ^{32}P-, 5′-end-labeled oligonucleotide, SX27 (5′ CTT-CAC-TCT-CTT 3′), was annealed to a 51-mer template containing one internally incorporated CDV molecule (5′ TGA-CCA-TGT-AAC-AGA-GAG-HAA-GAG-AGA-GAG-AAG-AGA-GTG-AAG-GTG-GTA-GTT; CDV is abbreviated as H [lane 2]). The reaction in lane 1 used a natural 51-mer template containing dCMP at position 19 (from the 5′ end) of the template instead of CDV as shown in the above sequence. A dideoxy sequencing ladder of the regular 51-mer template was used as a size marker. The primer extension reaction was carried out in a 10-μl reaction mixture containing 1 pmol of the primer-template complex, 20 mM Tris (pH 8.0), 10 mM $MgCl_2$, 0.2 mg of BSA per ml, 0.4 mM DTT, 90 mM $(NH_4)_2SO_4$, and 100 μM each deoxynucleoside triphosphate. To start the reaction, 0.12 U of HCMV DNA polymerase was added. After 2 h of incubation at 37°C, samples were analyzed by 15% polyacrylamide–8 M urea gel electrophoresis.

DISCUSSION

HCMV DNA polymerase exhibits fidelity when incorporating CDV into the DNA. The incorporation of a single molecule of CDV causes DNA synthesis to slow down by 31%. The disappearances of 18-mer primers following incorporation of dCTP or CDVpp are similar, indicating that the rate of incorporation of CDV into the DNA is similar to the rate of incorporation of dCMP by HCMV DNA polymerase. Thus, the presence of an acyclic phosphonate moiety in CDV does not affect the rate of CDV incorporation into DNA. These data are consistent with the report (15) that the V_{max} values (the V_{max} values for incorporating dCTP and CDVpp into DNA are 0.39 ± 0.08 and 0.27 ± 0.09 pmol/min/U [means ± standard deviations], respectively) for the incorporation of dCTP and CDVpp by HCMV DNA polymerase are similar. There are, at minimum, four kinetically defined steps in DNA polymerization: binding of template-primer, binding of deoxynucleoside triphosphate, incorporation of nucleotide into the 3′ end of the primer, and translocation of the newly formed 3′ end primer-template at the active site of the polymerase to complete the catalytic cycle. The rate-limiting step is the elongation of a 20-mer. These data also indicate that the translocation of the primer containing one molecule of CDV (19-mer [Fig. 3, lanes 7 to 11) at the 3′ terminus and the incorporation of the next incoming nucleotide (formation of a 20-mer) are at normal rates.

The incorporation of two consecutive CDV molecules prohibits the DNA from further elongating, which may be due to distortion of the primer end. This distortion may result in one or all of the following: primer end dissociation from the active site, very slow translocation of the primer end at the active site, and inability of the enzyme-primer-template complex to bind the next incoming deoxynucleoside triphosphate.

The incorporation of two CDV molecules separated by one molecule of natural nucleotide also drastically slows down DNA elongation. Some slight differences in the accumulation of elongating DNA primers, depending on the identity of the intervening nucleotide, were observed (Fig. 4). This indicates the potential for sequence-dependent inhibition of DNA elongation by CDVpp. Also, some sequence-dependent inhibition of DNA elongation was observed during the incorporation of

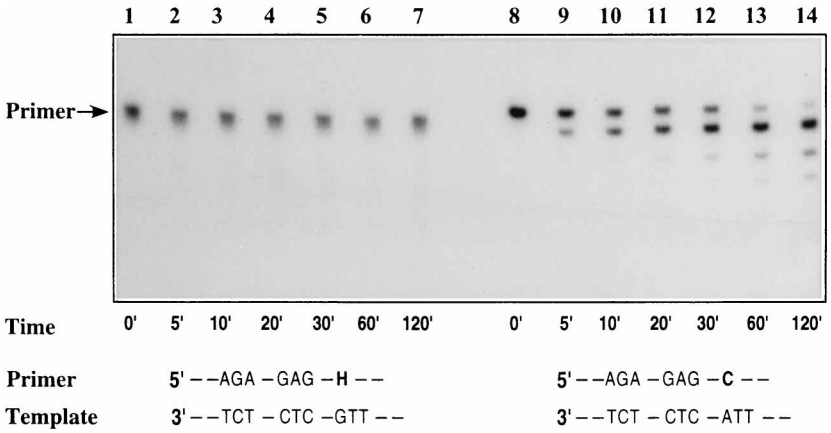

FIG. 7. Exonuclease activity of HCMV DNA polymerase on a primer containing one molecule of CDV or dCMP at the 3′ terminus hybridized to a template. Primer-template complex A (lanes 1 to 7) contained a 19-mer ^{32}P-, 5′-end-labeled oligonucleotide (5′ TGA-CCA-TGT-AAC-AGA-GAG-H 3′; CDV is abbreviated as H) annealed to a 36-mer template, SX2 (5′ TCT-CTT-CTC-TCT-CTC-TTG-CTC-TCT-GTT-ACA-TGG-TCA 3′). Primer-template complex B (lanes 8 to 14) contained a 19-mer ^{32}P-, 5′-end-labeled oligonucleotide (5′ TGA-CCA-TGT-AAC-AGA-GAG-C 3′) annealed to a 36-mer template, SX7 (5′ TCT-CTT-CTC-TCT-CTC-TTA-CTC-TCT-GTT-ACA-TGG-TCA 3′). Reactions were carried out in a 20-μl mixture containing 20 mM Tris (pH 8.0), 10 mM $MgCl_2$, 0.2 mg of BSA per ml, 0.4 mM DTT, 90 mM $(NH_4)_2SO_4$, and 0.24 U of HCMV DNA polymerase. For lanes 1 to 7, 0.24 pmol of primer-template complex A was used; for lanes 8 to 14, 0.32 pmol of primer-template complex B was used. Reactions were carried out at 37°C, and samples were taken at the indicated times (in minutes) from each reaction mixture for analysis by 15% polyacrylamide–8 M urea gel electrophoresis.

two molecules of CDV separated by two molecules of natural nucleotide (Fig. 5).

The fidelity of DNA elongation is not disturbed after the incorporation of one molecule of CDV (Fig. 6A). However, the sequencing of DNA containing one single molecule of internally incorporated CDV reveals the incorporation of either dA or dG complementary to CDV on the template (Fig. 6A). The misincorporation of dA complementary to CDV may be related to the use of Sequenase (T7 polymerase). Several attempts to synthesize a DNA fragment complementary to the template containing one single molecule of internally incorporated CDV for sequencing were made, using HCMV DNA polymerase. None of these attempts were successful, due to the greatly decreased rate of DNA synthesis when CDV is in the template (Fig. 6B, lane 2). It may be worthwhile to explore other DNA polymerases for their fidelity of incorporation utilizing CDV template.

The synthesis of DNA with a template containing a single internally incorporated CDV molecule causes the accumulation of elongating DNA complementary mainly to the nucleotides immediately before CDV, at CDV, and immediately after CDV in the template (Fig. 6B). Interestingly, the degree of inhibition is much larger with a template containing CDV than with a template incorporating CDV into a nascent strand. The exact reasons for this decreased rate of DNA synthesis with a template containing an internally incorporated CDV molecule are unknown.

We describe here a novel and unique effect of CDV on DNA synthesis catalyzed by HCMV DNA polymerase. In summary, these data indicate that CDV has several different mechanisms of inhibition. Importantly, we observed that in addition to inhibiting DNA synthesis, the 3′-to-5′ exonuclease activity of HCMV DNA polymerase does not remove the incorporated CDV from the elongating primer end. In addition to these multiple mechanisms of inhibition, the intracellular persistence of CDVpp and the prolonged half-life in plasma (tissue) may also account for the long-lasting antiviral activity of CDV.

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